# Conifer genetic engineering: Particle bombardment and *Agrobacterium*-mediated gene transfer and its application in future forests

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**Abstract:** Many important advances in forest biotechnology have been made. The use of genetic transformation and the applications of transgenic trees in modern forestry is now an important field. Two basic methodologies particle bombardment and *Agrobacterium*-mediated transformation have been used on conifers. However, routine procedures exist for only a limited number of conifers. As a result only a few species have been successfully transformed into stable transgenic plants. The use of a particle bombardment has been more successful and transgenic plants have been produced in *Picea abies, Picea glauca, Picea mariana*, and *Pinus radiata*, although the level of production of stable transgenic plants is lower than that of *Agrobacterium*. At present, breeding programs have been directed toward improving bole shape, growth rate, wood properties, and quality, as well as toward improving root and shoot performance, pest resistance, stress tolerance, herbicide resistance, and ability to resist stresses, which will drive forestry to enter a new era of productivity and quality. This article provides a brief overview of the current state of knowledge on genetic transformation in conifers.

**Keywords:** Genetic engineering; Particle bombardment; *Agrobacterium*; Conifer

## Instruction

Trees have traditionally not been the subject of biotechnology based breeding programs because of their large size and long life span. Long lag times between transformed seedling and the life stage at which the researcher can judge the outcome of his work by characterizing the adult tree and the large areas of land required to produce an adequate number of individuals are two features in genetic improvement of conifers. Genetic engineering techniques can selectively add a single trait to plants while still maintaining all of the derived traits in the parental lines. Since the regenerated transgenic plants was obtained in 1984 (Birch 1997), stable transformation has been developed for a lot of forest trees and used to transfer economically interesting genes conferring traits such as virus, insect, and herbicide resistance (Birch 1997; Walter *et al.* 1999). However, genetic transformation of conifers has only been reported recently and is restricted to a few species (Wenck *et al.* 1999; Ellis *et al.* 1993). Several infectious *Agrobacterium* strains that resulted in tumor development in numerous coniferous species have identified (Clapham and Ekberg 1986; Sederoff *et al.* 1986; Stomp *et al.* 1990; Morris *et al.* 1989). However, there are only a few reports of the regeneration of a stable transformation conifer using *Agrobacterium* (Ellis *et al.* 1993; Walter et al. 1998; Charest *et al.* 1996), and a few

1998; Charest *et al.* 1996), and a few reports of stable transformation by particle bombardment (Huang *et al.* 1991; Wenck *et al.* 1999; Levee et al. 1997), mainly due to the lack of a suitable regeneration procedure (Tang *et al.* 1998). With the various gene transfer methods currently available, simple placement or transfer of DNA into a plant cell is no longer a limiting factor. However, both mechanism for DNA transfer to plant cell and targeting of the DNA to a particular within a complex tissue or organ competent for regeneration is major limitation. Moreover, tissue culture methods that complement gene transfer technology do not exist for most conifers. Even when a plant regeneration method does exist for a particular conifer, the identity of those cells that divide to form new plants is often unknown. Since the aim is usually to produce trees with better wood properties, which can only be evaluated in the adult tree, there exists a long lag time between the actual work of transformation and the discovery of success or failure (Loopstra *et al.* 1990; Sambrook *et al.* 1989). The lack of knowledge of the genetic maps of conifers means that there are few genes that have been characterized well enough to be used in transformation experiments. The genes that have been studied have mainly been from novel phenotypes or for tolerance to various stresses. In this presentation, I provide a brief overview of the current state of knowledge on genetic transformation in conifers involved in T-DNA transfer process, *Agrobacterium*-mediated transformation, transformation via particle bombardment, and transformation using electroporation, where biotechnology is being used to improve tree production or usability.

## T-DNA transfer processes

Agrobacterium-mediated transformation is the best method in higher plant transformation. The integration pattern for foreign genes introduced via Agrobacterium-mediated transformation is in general striking different from the pattern resulting from particle bombardment of plant cells (Stachel et al. 1985; Sheikholeslam and Weeks 1987). The process of foreign gene transfer from Agrobacterium into plant cell includes several essential steps: a) bacterial colonization, b) induction of bacterial virulence system, c) T-DNA processing, d) bacterial attachment, e) generation of T-DNA transfer complex, f) T-DNA transfer and nuclear targeting, and g) integration of T-DNA into plant genome (Sheng and Citovsky 1996) (Fig. 1). Bacterial colonization is an essential step in tumor induction, and it takes place when Agrobacteriun is attached to the plant cells surface. The polysaccharides of the bacterium cell surface are proposed it play an important role in the colonizing process because the bacterial attachment could be prevented when lipopolysaccharides solution from virulent strains is applied to the plant tissue before interaction with virulent bacterium (James et al. 1993; Godwin et al. 1991). Research results from transposon insertion mutant show that the chromosomal 20 kb att locus contains the genes required for successful bacterium attachment to the plant cell. Genes that played at att left side are involved in molecular signaling elements, while the right side genes are likely to be responsible for the synthesis of fundamental components. Induction of bacterial virulence system and the T-DNA transfer are mediated by products encoded by the 30-40 kb vir region of the Ti plasmid. This region is composed by at least six essential operons: VirA, VirB, VirC, VirD, VirE, VirG, and two non-essential operons: VirH, VirF. VirA, VirG, and VirF have only one gene; VirE, VirC, and VirH have two genes; virD has four genes, and VirB has eleven genes. Both VirA and VirG are constitutively expression operons and code for s two-component system activating the transcription of the other vir genes. Vir A is a transmembrane bimeric sensor protein that detects signal molecules including phenolic compounds released from wounded plant tissue such as acetosyringone, acidic pH, and some monosaccharides which act sinergistically with phenolic compounds (Sheng and Citovsky 1996). Activated VirA has the capacity to transfer its phosphate to a conserved separate reside of the cytoplasmic DNA binding protein Vir G. Vir G functions as a transcriptional factor regulating the expression of vir genes when it is phosphorylated by VirA. The activation of vir genes produces the generation of single- stranded molecules representing the copy of the bottom T-DNA strand. Any DNA placed between T-DNA borders can be transferred to the plant cell, as single strand DNA, and integrated into the plant genome. The protein VirD1 and VirD2 play a key role in recognizing the T-DNA border sequences and nicking the bottom strand at each border. The nick sites are assumed

as the initiation and termination sites for T-DNA strand recovery. Extensive mutation or deletion of the right T-DNA border is followed by almost completely loss of T-DNA transfer capacity, which at the left border results in lower transfer efficiency (Sheng and Citovsky 1996). Plant DNA is subsequently cut at the 3'-end position of type gap by endonuclease, and the first nucleotide in the top plant DNA strand. Then the 3'-end of the T-strand joins the nicks in the bottom plant DNA strand Once the introduction of the T-strand in 3'-5' strand of the plant DNA is completed, a torsion followed by a nick in to the opposite plant DNA strand is produced. This activates the repair mechanism of the plant cell and the complementary strand is synthesized by using the early inserted T-DNA strand as a template (Sheng and Citovsky 1996).
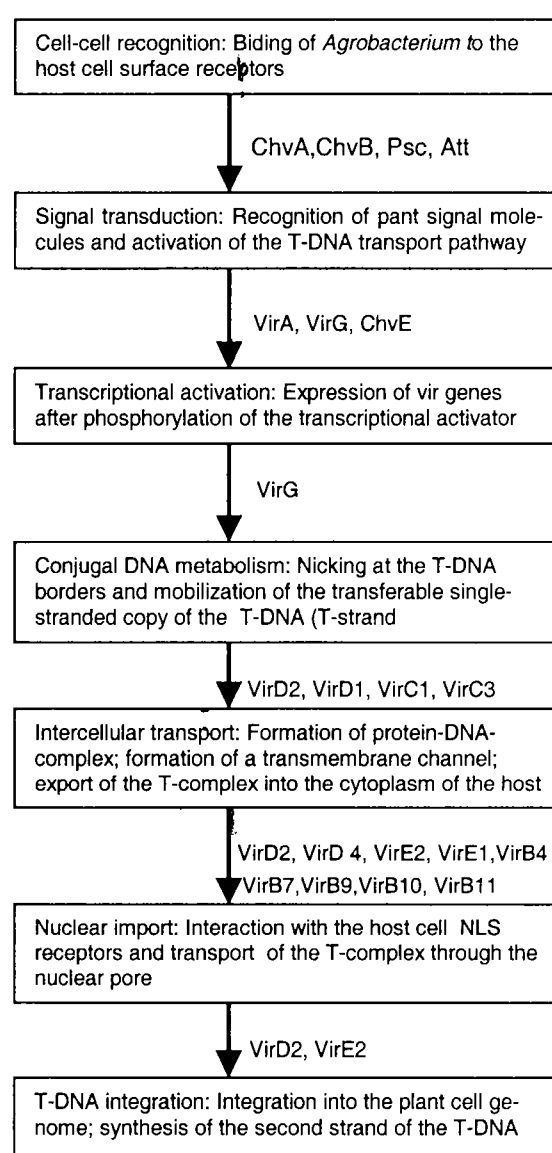
Cell-cell recognition: Biding of Agrobacterium to the host cell surface receptors

ChvA,ChvB, Psc, Att

Signal transduction: Recognition of pant signal molecules and activation of the T-DNA transport pathway

VirA, VirG, ChvE

Transcriptional activation: Expression of vir genes after phosphorylation of the transcriptional activator

VirG

Conjugal DNA metabolism: Nicking at the T-DNA borders and mobilization of the transferable single-stranded copy of the T-DNA (T-strand

VirD2, VirD1, VirC1, VirC3

Intercellular transport: Formation of protein-DNA-complex; formation of a transmembrane channel; export of the T-complex into the cytoplasm of the host

VirD2, VirD 4, VirE2, VirE1,VirB4
VirB7,VirB9,VirB10, VirB11

Nuclear import: Interaction with the host cell NLS receptors and transport of the T-complex through the nuclear pore

VirD2, VirE2

T-DNA integration: Integration into the plant cell genome; synthesis of the second strand of the T-DNA

**Fig. 1 Sequential processes of T-DNA transfer during Agrobacterium infection**

## Agrobacterium -mediated transformation of conifers

Agrobacterium is a soil bacterium that can genetically transform plant cells with transfer DNA (T-DNA) from a tumor- inducing plasmid (Ti plasmid) with the resultant production of a crown gall. Agrobacterium-mediated or direct transformation methods have been used for more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medical, fruit tree, and pasture plants (Birch 1997). However, efficient methodologies of Agrobacterium-mediated gene transfer have been established mainly for dicotyledonous plants. Agrobacterium-mediated has been applied to more than ten coniferous species (Table 1).

**Table 1. Agrobacterium-mediated transformation in conifers**

| Species | Agrobacterium strains* | Plasmid vectors | Gene expression | References |
|---|---|---|---|---|
| Abies nordmanniana | A.t.C58/pV3304/pV3851/pV2298 | C58/pV3304/pV3851/pV2298 | Tumor formation | Clapham et al. 1986 |
| Abies procera | A.t.A136(pTiEU6)/K27/B3.73/K41 | A136(pTiEU6)/K27/B3.73/K41 | Gall formation | Morris et al. 1989 |
| Larix decidua | A.r.pRi11325 | pRi11325 | Stable expression | Huang et al. 1991 |
| Larix decidua | A.r.11325 | pCGN1133/pWB139 | Stable expression | Shin et al. 1994 |
| Larix decidua | A.r.ATCC11325 | ATCC11325 | Tumor formation | Diner et al. 1987 |
| Larix kaempferi × L.decidua (hybrid larch) | A.t C58/pMp90/pMrKE70Km | p35S-nptII-p19s-nptII | Stable expression | Levee et al. 1997 |
| Larix laricina | A.r.A4/R100 | A4/pRiA4b | Stable expression | McAfee et al.1993 |
| Libocedrus decurrens | A.t/C58/M2/73 | C58/M2/73 | Gall formation | Stomp et al. 1990 |
| Picea abies | A.t. EHA105, A.t. LBA4404, GV3101 | pWWS006 | Stable expression | Wenck et al. 1999 |
| Picea engelmanni | A.t.A281/W2/73 | pEND4K/pLUX2 | Gall formation | Ellis et al. 1989 |
| Picea glauca | A.t.A281/W2/73 | pEND4K/pLUX2 | Gall formation | Ellis et al. 1989 |
| Picea sitchensis | A.t.A281/W2/73 | pEND4K/pLUX2 | Gall formation | Ellis et al. 1989 |
| Pinus banksiana | A.r. A4/R100 | A4/pRiA4b | Stable expression | McAfee et al.1993 |
| Pinus eldarica | A.t. U3 | U3 | Gall formation | Stomp et al. 1990 |
| Pinus elliottii | A.t.A281/542/C58/M2/73/U3 | A281/542/C58/M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pinus halepensis | A.r.LBA9402 | p35SGUSINT | Stable expression | Tzfira et al. 1996 |
| Pinus jeffreyi | A.t.C58 | C58 | Gall formation | Stomp et al. 1990 |
| Pinus lambertiana | A.t.Bo542/A28/Bo542kanr | pTiBo542/pEND4 | Stable expression | Loopstra et al. 1990 |
| Pinus lambertiana | A.t.A281/542/C58/M2/73/U3 | A281/542/C58/M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pinus monticola | A.r.A4/R100 | A4/pRiA4b | Stable expression | McAfee et al.1993 |
| Pinus pinea | A.t.EHA105,GV3850, A.t.LBA4404, C58 | p35SGUSint | Transient expression | Humara et al. 1999 |
| Pinus ponderosa | A.t.A136(pTiEU6)/K27/B3.73/K41 | A136(pTiEU6)/K27/B3.73/K41 | Gall formation | Morris et al. 1989 |
| Pinus radiata | A.t C2/74/542 | C2/74/542 | Gall formation | Bergmann . 1992 |
| Pinus radiata | A.t.A281/542/C58/M2/73/U3 | A281/542/C58/M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pinus taeda | A.t. M2/73/U3 | M2/73/U3 | Gall formation | Sederoff et al. 1986 |
| Pinus taeda | A.t.A281/542/C58/M2/73/U3 | A281/542/C58/M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pinus taeda | A.t. EHA105, A.t. LBA4404, GV3101 | pWWS006 | transient expression | Wenck et al. 1999 |
| Pinus virginiana | A.t. 542/ M2/73/U3 | 542/ M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pinus sylvestris | A.t. 542/ M2/73/U3 | 542/ M2/73/U3 | Gall formation | Stomp et al. 1990 |
| Pseudotsuga menziesii | A.t.A281/W2/73 | pEDD4K/pLUX2 | Gall formation | Ellis et al. 1989 |
| Pseudotsuga menziesii | A.t.A136(pTiEU6)/K27/B3.73/K41 | A136(pTiEU6)/K27/B3.73/K41 | Gall formation | Morris et al. 1989 |
| Pseudotsuga menziesii | A.t.A281/542 | A281/542 | Gall formation | Stomp et al. 1990 |
| Tsuga heterophylla | A.t.A136(pTiEU6)/K27/B3.73/K41 | A136(pTiEU6)/K27/B3.73/K41 | Gall formation | Stomp et al. 1990 |

* A.t.: Agrobacterium tumefaciens; A.r.: Agrobacterium rhizogenes

These studies demonstrated some important factors for the application of Agrobacterium for gene transfer in coniferous species, such as bacterial strains, vectors, selection marker genes and selection agents, promoters, genotypes of plants (Bergmann and Stomp 1992; Ellis et al. 1989; Diner and Karnosky 1987; DeCleene and Deley 1976). Physiological status various conditions of tissue culture, and developmental stage of the tissue inoculated. According to Levee et al. (1997) one to two transformation events per 100 co-cultivated embryogenic tissues were yielded in hybrid larch using Agrobacterium tumefaciens. The addition of 100 (M coniferal alcohol during the co-cultivated step increased the frequency of kanamycin-resistant tissues where other substances know to be virulence inducers such as acetosyringone and syringaldehyde did not seem to have any effect. Different bacterial strains (C58,

pMB90, EHA101, and LBA4404) with various chromosomal backgrounds were tested, but none of them increased significantly the frequency of kanamycin -resistant tissues. Tzfira et al (1996) used Agrobacterium rhizogenes strain LBA9402 to transform Pinus halepensis embryos, seedlings and shoots. Mature embryos exhibited susceptibility to the Agrobacterium with more than 85% showing considerable transient GUS expression in the radicle. GUS expression was also observed in cotyledons, but at lower rate of about 24% of the embryos (1-5 spots/embryo).

Great Advance has been made in Agrobacterium-mediated genetic transformation in conifers. Reporter genes and important trait genes have been used in conifers by Agrobacterium-mediated transformation. Agrobacterium-mediated gene transfer has been reported in more than twenty coniferous species (Table1) involving in Abies, Larix, Libocedrus, Picea, Pinus, Pseudotsuga, and Tsuga. These studies demonstrated that some factors were important for the application of Agrobacterium for gene transfer in conifers. They include: a) Variation in infectively by different Agrobacterium strains; b) infectivity is different for different conifers and genotypes; c) infectivity is dependent on the physiological statue and developmental stage of the tissue inoculated. Transgenic regenerated plantlets were obtained from transformed tissue cultures of Larix decidus and Pinus halepensis (Huang et al. 1991; Tzfira et al. 1996) infected by Agrobacterium rhizogenes, as well as from embryogenic tissue of hybrid larch transformed by Agrobacteriun tumefaciens (Levee et al. 1997). Humara et al. (1999) reported the transfer and expression of foreign chimeric genes in cotyledons of Pinus pinea. They transformed embryos by Agrobacterium tumefaciens EHA 105 harboring the plasmid p35SGUSint and found that 49.7% of cotyledons showed a diffuse blue staining 7 days after infection. Recently, transgenic plants of Picea abies have produced by using embryogenic callus as targeted tissues (Wenck et al. 1999).

## Transformation via particle bombardment

Particle bombardment-mediated transformation can be used to regenerate whole plants (Charest et al. 1993; Ellis et al. 1991; Walter et al. 1994). The ability to deliver foreign DNA into regenerated cells, tissues, and organs appears to provide the best method for achieving truly genotype-independent transformation by passing Agrobacterium host specificity and tissue-culture-related regeneration difficulty. There is no biological limitation to the actual DNA delivery process, consequently genotypes is not a limiting factor. The recent advances in the transformation of other crop plants with particle bombardment have demonstrated that DNA can be inserted into virtually any tissue and cell which is impacted by the particle (Bommineni et al. 1993). Indeed, foreign genes were expressed in all conifer tissues exposed to particle bombardment including embryos, seedlings, megagametophytes, xylem, pollen, needles, buds,

cell suspension cultures, embryogenic callus, cell aggregate cultures, and roots tested thus far (Walter et al. 1999). While almost all of this expression was transient, it yielded valuable information on factors involved in the expression of introduced genes in the various tissues competent for regeneration. Parameters which were found to influence successful particle and DNA delivery into regenerable tissue of plants included condition of the explant prior to bombardment, environmental factors including temperature and humidity, and influences of the parameters on transient activity of the marker genes, additional important parameters also included depth of particle penetration and degree of tissue damage as a function of accelerating force and timing of selection. In Pinus, transient expression of green fluorescent protein gene in embryogenic masses of Pinus strobus via particle bombardment has been achieved (Tian et al. 1997). Transient expression of β-glucuronidase (GUS) gene (Jefferson et al. 1987) in cotyledon cells of Pinus taeda by particle bombardment has been reported (Stomp et al. 1991), and results demonstrated that microprojectile bombardment had potential for the production of transgenic plants in pine. The transient regenerated pine was obtained by transforming embryogenic tissues of Pinus ratiada using a biolistic particle delivery system (Walter et al. 1998), and more than 150 transgenic Pinus radiata plants were produced for 20 independent transformation expression with four different embryogenic clones.

The expression of foreign genes in conifer was observed on meristematic cells that have the ability to rapidly divided and have a high rate of metabolic activity. Cells with high metabolic activity would likely be active in endogenous gene expression with all the functions for gene transcription and translation actively expressed. So genes introduced into these cells would also have a higher probability of being expressed, since a) DNA replication may aid in the incorporation of foreign DNA; and b) a certain phase (S phase) in the cell cycle may be a prerequisite for DNA integration in to the genome. Transformation of conifers by particle bombardment included Larix laricina (Klimaszewska et al. 1997), Larix decidua (Duchesne et al. 1993), Picea abies (Robertson et al. 1992), Picea glauca (Elli et al. 1993), Picea mariana (Charest et al. 1996; Duchesne et al. 1991), Pinus radiata (Walter et al. 1998), Pinus strobus (Tian et al. 1997), Pinus taeda (Stomp et al. 1991), and Pseudotsuga menziesii (Goldfarb et al. 1991) (Table 2). Stable transformation of Picea glauca by particle bombardment (Table 2) and transgenic regenerated plantlets were obtained by transforming embryogenic cultures, and incorporation of the introduced genes into the genome was confirmed by PCR and Southern blot analysis of embryogenic callus and regenerated transformed plants, as well as spruce budworm feeding trials with transformed tissues (Ellis 1993). Stable transformation of Norway spruce tissue had been obtained following bombardment of mature somatic embryos with pRt99Gus (Robertson, 1992).

Stable transformation of *Picea mariana* by particle bombardment had finished by Charest (1996). An efficient particle bombardment has been developed by stably transforming *Picea abies* several embryogenic tissue lines. transgenic *Picea abies* plants from nine independent transformation events were recovered and are growing in a greenhouse for future investigation (Walter et al. 1999). In addition, pollen grain transformation by particle bombardment has been tested successful. The grains are trans-

formed and a cone from a selected female is pollinated. The production of transgenic individuals in this way avoids the need for in vitro regeneration. Normal cone development has been reported in *Pinus aristata*, *Pinus griffithii*, *Pinus monticola*, Norway spruce, and Scots pine (Table 2), and transgenic plants have not yet to be produced and regeneration of transformed conifer embryos is proving to be difficult (Fernando et al. 2000).

**Table 2. Transformation of conifers by particle bombardment**

| Species | Bombaeded tissues | Plasmid vectors | Gene expression | References |
|---|---|---|---|---|
| *Larix decidua* | Embryogenic cells | pRT99GUS | Transient expression | Duchesne et al. 1993 |
| *Larix leptolepis* | Embryogenic cells | pRT99GUS | Transient expression | Duchesne et al. 1993 |
| *Larix leptoeuropae* | Embryogenic cells | pRT99GUS | Transient expression | Duchesne et al. 1993 |
| *Larix (eurolepis × hybrid larch)* | Embryogenic cells | pRT99GUS | Transient expression | Duchesne et al. 1993 |
| *Larix laricina* | Embryogenic cultures | pRT66gus, pRT55gus, and pRT99gus | Stable expression | Klimaszewska et al. 1997 |
| *Picea abies* | Somatic embryo cultures | pRT99GUS | Stable expression | Robertson et al. 1992 |
| *Picea abies* | Embryogenic tissue | pCW122 | Stable expression | Walter et al. 1999 |
| *Picea glauca* | Embryogenic cultures | pTUBT41100 | Stable expression | Ellis et al. 1993 |
| *Picea glauca* | Embryonal masses | p35S-GFP, mGFP | Transient expression | Tian et al, 1997 |
| *Picea glauca* | Embryos and seedlings | pUC19 | Transient expression | Ellis et al. 1991 |
| *Picea glauca* | Embryogenic suspension cultures | pBI426 | Stable expression | Bommineni et al. 1993 |
| *Picea mariana* | Pollen, embryonal masses, suspension cultures, and somatic embryos | p35S-GFP, mGFP | Transient expression | Tian et al. 1997 |
| *Picea mariana* | Embryogenic callus | pRt99GUS, pBM113kp | Transient expression | Duchesne et al. 1991 |
| *Picea mariana* | Embryogenic cell lines | PRt99GUS, pBI426 | Stable expression | Charest et al. 1996 |
| *Pinus aristata* | Pollen cones | pBI221 | Transient expression | Fernando et al. 2000 |
| *Pinus griffithii* | Pollen cones | pBI221 | Transient Expression | Fernando et al. 2000 |
| *Pinus monticola* | Pollen cones | pBI221 | Transient expression | Fernando et al. 2000 |
| *Pinus radiata* | Embryogenic cultures | pEmuGN, pCW103, p40CSD35SIGN, pCW5, pCW6, and pCW122 | Transient expression | Walter et al. 1994 |
| *Pinus radiata* | Suspension cells | PBI221, pCAMVLN | Transient expression | Campbell et al. 1992 |
| *Pinus radiata* | Embryogenic tissues | PRC101, pCW122 | Stable expression | Walter et al. 1998 |
| *Pinus strobus* | Embryonal masses | p35S-GFP, mGFP | Transient expression | Tian et al. 1997 |
| *Pinus taeda* | Cotyledons | pBI221 | Transient expression | Stomp et al. 1991 |
| *Pseudotsuga menziesii* | Cotyledons | pTVBTGUS | Transient expression | Goldfrab et al. 1991 |

Transformation using electroporation as a method of direct gene transfer has potential for genetic improvement and studies of gene structure and function in forest trees. This is especially attractive when used in conjunction with protoplasts that are capable of regeneration to somatic embryos or plantlets (Bekkaoui et al. 1998; Wilson et al. 1989; Tautorus et al. 1989). Because electroporation avoids the host-range limitation of *Agrobacterium*-mediated transfer methods, it has the further advantage of being useful for the rapid evaluation of the functionality of plasmid construction, for assessing transient gene expression,

and stable transformation. Electroporation has been utilized to transfer gene into protoplasts isolated from embryogenic cell culture of *Picea glauca* (Bekkaoui et al. 1988), *Picea mariana* (Tautorus et al. 1989), *Pinus taeda* (Gupta et al. 1988), *Pseudotsuga menziesii* (Dandekar et al. 1987), and *Larix x eurolepis* (Charest et al. 1991), and from non-embryogenic cultures of *Pinus radiata* (Campbell et al. 1992) and *Pinus banksiana* (Tautorus et al. 1989), These experiments have demonstrated that the 35S cauliflower mosaic viral (CaMV 35S) and the nopaline synthase (nos) promoter function in conifers tissues. They also show

that commonly used reporter genes, such as firefly luciferase (Luc) gene and β-glucuronidaes (GUS) gene, can be used to assess gene activity in conifer protoplast. Bekkaoui et al. (1990) reported that the level of foreign gene activity in electroporation Picea glauca, Picea mariana, and Pinus banksiana protoplast is dependent on the promoter transferred, electroporation conditions, as well as on the target cell line under investigation. Gupta et al. (1988) reported that the viability of protoplast was reduced from 90% to 45%-55% after electroporation, the transient expression of the luc gene was detected in protoplasts surviving 36 h after electroporation. Gene expression was improved by the addition of polyethylene glycol (PEG) to the electroporation mixture. According to Tautorus et al. (1989), transient expression of the chloramphenical acetyltransferase (CAT) gene in electroporated Picea mariana and Pinus banksiana protoplasts was affected by the cell lines used, by voltage, temperature, and by the plasmid concentration and conformation. Increasing the plasmid DNA concentration resulted in higher levels of transient CAT expression. Linearized plasmid gave 2.5 times higher levels of CAT enzyme activity than circular in Pinus banksiana. A heat shock treatment of protoplasts for 5 min at 45°C resulted in enhanced CAT gene expression for Picea mariana and Pinus banksiana. Because of the difficulty in plant regeneration from conifer protoplasts, transformation using electraporation is mainly used to study transient expression of gene and factors influencing transgene expression.

## Application of genetic engineering technology in future forests

Application of genetic engineering technology in future forests includes: 1) The alteration of tree form and performance; 2) Insect resistance and herbicide resistance; 3) Abiotic stress tolerance; 4) Modifying lignin content and composition. Insect resistance and herbicide resistance and modifying lignin content and composition have being widely studied in higher plants (Baucher et al. 1996; Bowler et al. 1991; Comai et al. 1985). Insect resistance involves the introduction of a gene coding for Bt toxin from Bacillus thuringensis. The Bt toxin inhibits the insect's digestive pathways. This gene has been successfully transformed into several species including larch and conifers with varying degrees of success. Herbicide resistance has allowed the use of more efficient herbicides without concern for plant health in forestry, especially for higher intensity plantation systems. Herbicide resistance would be very useful especially in younger trees, where competition from weeds is the greatest (Shin et al. 1994). An example of herbicide resistance being introduced into a tree species is the work of Fillatti et al. (1987). A binary oncogenic strain of Agrobacterium tumefaciens was introduced into a poplar clone. The strain carried a mutant gene (aroA), which codes for 5-enolpyruvylshikimate 3-phosphate (EPSP)

synthase that is less susceptible to the herbicide glyphosate (N-phosphonomethylglycine) than the wild type protein. Transformation was performed by co-cultivating leaf segments with A. tumefaciens. Leaves from sterile cultures were incubated on plates for 24 hours, then transferred to a broth culture containing A. tumefaciens for 30 minutes. The segments were then placed on a regeneration medium containing kanamycin. Using a method of Reiss et al. (1981), the NPT II activity of the plant tissue was measured to determine whether the tissue had been completely transformed. Analysis of the introduction of the aroA gene, coding for the EPSP synthase enzyme less susceptible to glyphosate, was carried out using western blot analysis after Comai et al. (1985). The resolved polypeptides were then transferred to filters that contained anti EPSP serum, washed, transferred to new filters with a labeling protein containing 125I, then exposed to x-ray film to obtain a picture of the results. Initial trials with the transformed plants have indicated glyphosate resistance. Attempts have been made to engineer tree species with genes capable of inducing tolerance to extreme environmental conditions. Plants have been engineered with antifreeze genes from fish and with genes altered lipid composition in their membranes in order to withstand extreme cold temperatures. Genes involved in drought tolerance are being sought and genes from bacteria that remove toxic metals from soil have being transformed into plants.

Lignin is the second most abundant organic compound on earth, and represents about 25% of the global wood biomass. Although lignin is an important compound for wood development it is an obstacle to efficient pulp and paper production as the lignin must be removed in order to extract the cellulose from the wood. This process is energy consuming and requires the use of polluting chemicals. It is of great interest to try and engineer trees to have a lower lignin component or a lignin type that is easily extracted without reducing tree growth rates or bole form. Lignins are a group of compounds formed from 3 precursors: 4-hydroxycinnamyl-alcohol (p-coumaryl alcohol), coniferyl alcohol, and sinapyl alcohol. Conifers could be engineered with lower lignin contents, or more syringyl lignin instead of the guaiacyl lignin. An example of tree lignin composition modification is the paper by Baucher et al. (1996). Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme in the production of lignin because it catalyzes the last step between the cinnamaldehydes and the three lignin precursor alcohols. The production of CAD in the plant is a good target for attempting to genetically engineer a tree with lower lignin content. Van Doorselaere et al. 1995 described the isolation of the CAD gene in a paper. The protocol used is said to be standard after that of Sambrook et al. (1989). DNA sequences were carried out using a Qiagen plasmid kit with the dideoxy chain termination procedure method after Sanger et al. (1977). The full CAD cDNA was isolated as a BamHI fragment and inserted in sense and anti-sense orientation into the pGSJ780A binary vector with a T-DNA

region having the cauliflower mosaic virus promoter and a NPT-II (kanamycin resistance) gene (Bowler et al. 1991). The use of both sense and anti-sense cDNA provides two treatments for the transformed poplar clones. The sense gene will have normal function and could increase CAD production, while the anti-sense gene will base pair with normal CAD and possibly reduce its activity (Leple et al. 1992). Transformation was accomplished by co-cultivating the poplar clone with the Agrobacterium containing the vector. Baucher et al. (1996) did not indicate how the poplar was prepared for regeneration or the conditions of growth and rooting after transformation. The results from CAD cDNA transformation seem promising for the improvement of biomass producing trees for paper pulp production.

## Conclusions

Particle bombardment and Agrobacterium-mediated transformation have been successfully for a wide range of conifers including Abies nordmaniana, Larix deciduas, Picea abies, Picea glauca, Picea mariana, Pinus radiata, Pinus taeda, Pinus elliottii, and Pseudotsuga menziesii. Production of genetically engineered conifers with commercially useful traits such as herbicide and insect and pathogen resistance has been conducted. On the base of understanding of fundamental developmental pathways that involved in wood quality and reproductive development, isolation and characterization of genes and promoters involved in lignin biosynthesis, cellulose deposition, flowering and other developmental processes have been tested. All of these will aim at the definition of the physical and chemical factors that control the differentiation of wood fibers in vitro. Further development of gene transfer technologies for conifers will allow the assessment of xylem specific genes and promoters and specific fungal resistance genes in transgenic conifers, and the production of herbicide resistant forest tree germplasm, and those will develop to form commercially applicable technologies. Genetic engineering program is focused on developing technology platforms and providing research services that lead to a better understanding of tree development and gene function. Forest research has been and firmly committed to playing a positively scientific role in the public debate concerning the application of genetic engineering technology in forestry and environmental biology. The techniques previously used for genetic transformation of agricultural plants are now finding their way into the engineering of forest tree species. At present, traditional breeding programs procedure at a slow rate due to long maturation times and the slow growth rate of trees, biotechnological approaches have the potential to provide significant improvement in tree growth and quality. If these problems can be addressed, forestry will enter a new era of productivity and quality.

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